AUROTHIOMALATE INHIBITS THE EXPRESSION OF mPGES-1 IN PRIMARY HUMAN CHONDROCYTES

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TUURE LAURI: AUROTHIOMALATE INHIBITS THE EXPRESSION OF mPGES-1 IN PRIMARY HUMAN CHONDROCYTES

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Avainsanat: DMARD, PGE_2, arthritis

Prostanoidit muodostuvat solukalvojen rasvahapoista syklo-oksygenaasientsyymien (COX-1 ja COX-2) katalysoimana. Prostaglandiini E_2 (PGE_2) on fysiologisten tehtäviensä lisäksi tärkeä tulehduksen ja tulehduskivun välittäjäaine. mPGES-1 on PGE_2:n muodostumista katalysoiva entsyymi, jonka ilmentyminen lisääntyy tulehdusreaktion yhteydessä.

Nykyisten tulehduskipulääkkeiden teho perustuu COX-entsyymien toiminnan estoon. Tämän seurauksena PGE_2:n lisäksi muidenkin prostanoidityyppien ilmentyminen vähenee, mistä aiheutuu COX-estäjille tyypillisiä haittavaikutuksia. On esitetty, että yhdisteillä, jotka selektiivisesti estäisivät mPGES-1:n aktiivisuutta tai ilmentymistä, olisi tulehduskipulääkkeiden kaltainen teho, mutta vähemmän haittavaikutuksia.

Tutkimuksen tavoitteena oli pystyttää malli, jossa voidaan tutkia mPGES-1:n ilmentymistä ihmisen rustosoluissa ja edelleen mitata markkinoilla olevien reumalääkkeiden vaikutuksia mPGES-1:n ilmentymiseen.

Rustokudosnäytteet saatiin tekonivelleikkauspotilailta. mPGES-1:n ilmentymistä tutkittiin käyttäen RT-PCR ja Western Blot-menetelmiä. Näytteiden PGE_2-pitoisuudet määritettiin käyttäen ELISA-menetelmää.

Viljellyt rustosolut ilmensivät mPGES-1-entsyymiä. Kun viljelmään lisättiin IL-1β:aa, mPGES-1:n ilmeneminen lisääntyi voimakkaasti. mRNA:n ilmentyminen lisääntyi 48 tunnin ajan, minkä jälkeen se alkoi vähentyä. Proteiinin ilmentyminen lisääntyi koko 96 tunnin seurannan ajan.

Tässä tutkimuksessa havaittiin että aurotiomalaatti vähensi mPGES-1:n ilmentymistä selvästi, kun taas muut tutkitut reumalääkkeet (metotreksaatti, sulfasalatsiini, hydroksiklorokiini) eivät vaikuttaneet mPGES-1:n ilmentymiseen rustosoluissa. Aurotiomalaatin vaikutus mPGES-1:n ilmentymiseen kliinisesti merkittävällä pitoisuudella (25 μM**)** oli verrattavissa deksametasoniin, jonka tiedetään vähentävän mPGES-1:n ilmentymistä useissa solutyypeissä. Aurotiomalaatti vähensi myös PGE_2:n pitoisuutta soluissa deksametasonin tavoin.

Löydöksen perusteella voidaan esittää uusi tulehdusreaktiota vaimentava mekanismi aurotiomalaatille. Tutkimuksen tuloksia voidaan hyödyntää jatkossa tutkittaessa mPGES-1:n ilmentymisen säätelyyn vaikuttavia tekijöitä ja kehitettäessä uusia anti-inflammatorisia lääkkeitä.

SISÄLLYSLUETTELO

INTRODUCTION

Prostaglandins are essential regulator molecules synthesized from arachidonic acid originating from cellular membranes. Arachidonic acid is metabolized to prostaglandin G_2 (PGG_2) and onwards to prostaglandin H_2 (PGH_2) by the constitutive cyclooxygenase 1 (COX-1) and by the inducible cyclooxygenase 2 (COX-2) enzyme which is up-regulated especially during inflammation (Figure 1).

Prostaglandin E_2 (PGE_2) is formed from PGH_2 by enzymes known as PGE synthases. Microsomal prostaglandin E synthase-1 (mPGES-1) is an inducible enzyme expressed in various human cell types (1) whereas the two other PGE_2 synthases, microsomal prostaglandin E synthase-2 (mPGES-2) and cytosolic prostaglandin E synthase (cPGES), are constitutively expressed and presumed to have a physiological role. mPGES-1, as well as COX-2 enzyme, is induced by inflammatory cytokines such as interleukin-1β (IL-1β), lipopolysaccharide (LPS) and tumour necrosis factor-α (TNF-α) (2,3). COX-2 and mPGES-1 are both overexpressed in connection with pathological processes including inflammation.

Non-steroidal anti-inflammatory drugs (NSAIDs) inhibit both of the COX enzymes suppressing the formation of PGH_2 and all of its terminal products including PGE_2. Inhibition of PGE_2 synthesis is regarded as the main mechanism of the therapeutic effects of NSAIDS. Unfortunately, the most common adverse effects of NSAIDs also appear to be mediated through reduced prostanoid production. Especially the cardiovascular side effects now known to be associated with the use of both non-selective and COX-2 selective NSAIDs are mediated through inhibition of the synthesis of prostanoids other than PGE_2 (3-5). A specific mPGES-1 inhibitor is therefore hypothesized to retain the therapeutic activity of NSAIDs with less adverse effects (5,6).

In addition to its physiological roles, PGE_2 is a vital mediator of inflammatory responses, pain and fever playing an important role in the mechanisms and symptoms of rheumatic diseases. mPGES-1 levels have previously been shown to be high in the synovial cells from patients with rheumatoid arthritis (RA) (7) and osteoarthritis (OA) (8). A convincing evidence of its pathophysiological role is also presented in an experimental model of RA in mPGES-1 knockout mice where the incidence

and severity of the disease, its clinical score and joint damage were reduced in knockout mice as compared to wild type controls (9). This suggests that inhibition of the pathological increase of mPGES-1 levels during arthritis might suppress the inflammatory activity and prevent / retard the progression of joint erosions.

The aim of the present study was to investigate the expression of mPGES-1 in activated primary human chondrocytes, and to study the effects of DMARDs methotrexate, sulfasalazine aurothiomalate and hydroxychloroquine, and of glucocorticoid dexamethasone on it.

MATERIALS AND METHODS

The study was approved by the Ethics Committee of Tampere University Hospital, Tampere, Finland and a written informed consent was obtained from the patients. Leftover pieces of OA cartilage from knee joint replacement surgery were received from Coxa Hospital for Joint Replacement, Tampere, Finland and processed as earlier described (10).

Isolated chondrocytes were plated at the density of 0.15 milj/ml in 24-well plates in DMEM culture medium with GIBCO GlutaMAX-I supplemented with penicillin (100 U/ml), streptomycin (100 µg/ml) and amphotericin B (250 ng/ml) containing 10% fetal bovine serum, and cultured at 37°C in a humidified 5% carbon dioxide atmosphere. After 24h incubation, the cells were treated with the compounds of interest. RNA or proteins were extracted and cell culture media collected after the desired time of incubation. RNA extraction and real-time RT-PCR, preparation of cell lysates for Western blot, and Western blot analysis were carried out as previously described (11).

Primer and probe sequences for human mPGES-1 were CACGCTGCTGGTCATCAAGA (forward), CCGTGTCTCAGGGCATCCT (reverse) and AGCCTCACTTGGCCCGTGATG (probe). Antibodies used in Western blot analysis were rabbit polyclonal antibody for mPGES-1 (AS-03031, Agrisera AB, Vännäs, Sweden; primary antibody) and goat anti-rabbit IgG-HRP (sc-2004, Santa Cruz, CA, USA; secondary antibody). Concentrations of PGE_2 in the cell culture medium samples were determined by ELISA (Cayman Chemical Co, MI, USA).

Cell culture medium and its supplements were from Invitrogen/Life Technologies (CA, USA), IL-1ß was purchased from R&D Systems Inc (MA, USA), dexamethasone, methotrexate and hydroxychloroquine were from Orion Corp. (Espoo, Finland), and all other reagents were obtained from Sigma Chemical Co. (MO, USA).

The results were analyzed with one-way ANOVA followed by Dunnett's post test by using InStat version 3.05 for Windows. Results are expressed as mean + standard error of the mean (SEM). P value lower than 0.05 was considered to indicate statistical significance.

RESULTS

mPGES-1 expression in unstimulated chondrocytes was low and a clear increase in its expression levels were found when IL-1β was introduced into the culture (Fig. 2). mPGES-1 mRNA expression increased up to 48 h and declined thereafter (Fig. 2A) whereas the protein expression continued to increase throughout the 96 h follow-up (Fig. 2B). As seen in Figure 2, the mPGES-1 expression in IL-1β –activated primary chondrocytes demonstrated relatively slow onset and long duration.

The effects of dexamethasone, aurothiomalate, sulfasalazine, methotrexate and hydroxychloroquine on IL-1ß –induced mPGES-1 expression were investigated. Aurothiomalate and dexamethasone significantly down-regulated mPGES-1 expression whereas sulfasalazine, methotrexate and hydroxychloroquine had no effect (Fig 3A). The effect of aurothiomalate on mPGES-1 mRNA expression was dose-dependent (Fig. 3B) and it was reflected as reduced mPGES-1 protein levels (Fig. 3C) and PGE_2 production (Fig. 3D).

DISCUSSION

In the present study, we measured the expression of mPGES-1 in primary human chondrocytes, and investigated the effects of DMARDs aurothiomalate, sulfasalazine, methotrexate and hydroxychloroquine, and dexamethasone on it. Interestingly, aurothiomalate was found to inhibit

the expression of mPGES-1 which is an original finding. The other DMARDs studied did not effect mPGES-1 expression while dexamethasone reduced mPGES-1 levels and PGE_2 production. The effects of aurothiomalate on mPGES-1 expression and PGE_2 production were dose-dependent, and a significant inhibition was achieved at clinically relevant concentration of 25 μM which can be maintained in serum during intramuscular aurothiomalate treatment (12). Further, aurothiomalate levels in synovial fluid are reported to correspond the serum levels during drug treatment (13).

It has previously been reported that glucocorticoids inhibit the expression of both mPGES-1 and COX-2 enzymes (2) and therefore dexamethasone was selected as a control compound when we evaluated the effects of disease-modifying antirheumatic drugs (DMARD) on the expression of mPGES-1. Methotrexate, sulfasalazine and hydroxychloroquine (triple therapy) were selected to this design because using them with a small dose of glucocorticoid as a combination therapy is evidently more effective treatment in RA than using a single DMARD with or without a small daily dose of glucocorticoid (14). The above mentioned combination is also recommended in the "Current Care Guidelines" in Finland as a primary treatment of an early active RA. TNF-alpha blockers which are nowadays widely used in the treatment of RA not responding adequately to traditional DMARDs, have been shown not to have an effect on mPGES-1 expression in synovial tissue (15) and therefore they were not included in this study.

Aurothiomalate is a traditional antirheumatic drug used in the treatment of RA since early 1930s (16). It is known to be an effective treatment for RA but its clinical usability is limited because of adverse effects. The molecular mechanisms of the anti-inflammatory and antirheumatic action of aurothiomalate are not known in detail. Aurothiomalate was reported to inhibit prostaglandin synthesis already in 1974 (17) and it was found to down-regulate the expression of COX-2 in murine chondrocytes by destabilizing its mRNA (18). Also, aurothiomalate was recently reported to enhance the expression of mitogen activated protein kinase phosphatase 1 (MKP-1) in murine and human chondrocytes (19) which can be linked to down-regulation of inflammatory genes. Findings in the present study introduce mPGES-1 as a novel target of aurothiomalate and extend our knowledge on the mechanisms of action of this DMARD.

The key role of mPGES-1 in pain and inflammatory responses has been convincingly demonstrated in experiments with mPGES-1 deficient mice. The knockout mice seem to develop normally but

they have attenuated inflammatory and pain responses, also in models of arthritis. Nephrotoxicity, skin reactions and gastrointestinal complications, side-effects of aurothiomalate treatment, have not been reported in mPGES-1 knockout mice (9) indicating that those adverse effects are most likely not mediated by down-regulation of mPGES-1.

PGE_2 is also an essential mediator of inflammatory pain. It has recently been found to induce and amplify pain perception by activating peripheral nociceptors via specific PGE_2-receptors, and also to modulate the activity of pain pathways in the spinal cord (20). The connection between different forms of pain and different PGE_2-receptor subtypes are currently under intensive research.

A selective inhibition of PGE_2 formation might be an effective manner to treat many types of pain with less adverse effects as compared to NSAIDs. Intensive research on mPGES-1 inhibitors has been ongoing since the enzyme was characterized, and many compounds with different molecular structures seem to inhibit it, but a specific inhibitor appliable for clinical use is yet to be discovered (5,21-24). Another approach to reduce PGE_2 production especially in inflammatory conditions might be down-regulation of mPGES-1 expression and according to the present findings that is achieved by using aurothiomalate but not with the other commonly used DMARDs.

In conclusion, the results of the present study introduce aurothiomalate as the first and so far the only DMARD found to be able to inhibit mPGES-1 expression. The effect is likely involved in the mechanisms of action for this gold containing DMARD in rheumatic diseases. These results are implicated in the regulatory mechanisms of mPGES-1 that are under intensive research to develop improved treatments for inflammation and inflammatory pain.

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FIGURE LEGENDS

Figure 1: Biosynthesis of prostaglandin E_2 and other prostanoids from arachidonic acid originated from cellular membranes. COX, cyclooxygenase; PGG_2, prostaglandin G_2; PGH_2, prostaglandin H_2; mPGES-1/2, microsomal prostaglandin E synthase 1/2; cPGES, cytosolic prostaglandin E synthase; PGE_2, prostaglandin E_2; PGD_2, prostaglandin D_2; $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$; PGI_2, prostaglandin I_2; TXA_2, thromboxane A_2.

Figure 2: The time-dependent expression of mPGES-1 enzyme in primary human chondrocytes in response to IL-1β.

A: Cells were incubated with IL-1β (100 pg/ml) and RNA was extracted at indicated time-points. mPGES-1 mRNA levels were analyzed with quantitative real-time reverse transcription–polymerase chain reaction (RT-PCR) and normalized against GAPDH. Untreated cells were used as control, and expression levels were quantified against them.

B: Cells were incubated with IL-1β (100 pg/ml) and proteins were extracted at indicated time-points. Untreated cells were used as control, and mPGES-1 expression levels determined by Western blotting were quantified against them. Actin was used as a loading control.

Values are mean + SEM expressed as fold increase from baseline (considered as 1) in arbitrary units, n=4-6, ** = $p < 0.01$.

Figure 3: The effects of dexamethasone and DMARDs on the expression of mPGES-1 and production of PGE_2 in primary human chondrocytes. The IL-1β-treated cells were considered as 100 % and the expression levels were quantified as percentages of them.

A and B: Cells were treated with IL-1β (100 pg/ml) and the compounds of interest for 48 h and RNA was extracted thereafter, subjected to RT-PCR and normalized against GAPDH. Untreated

cells were used as control, and expression levels were quantified against IL-1β –treated cells. Values are mean + SEM, n=4-11, ** = $p < 0.01$.

C: Cells were incubated with IL-1β (100 pg/ml) and the compounds of interest for 72 h, and proteins were extracted thereafter. Untreated cells were used as control, and mPGES-1 expression levels determined by Western blotting were quantified against IL-1β –treated cells. Actin was used as a loading control. Values are mean + SEM, n=8, ** = $p < 0.01$.

D: Cells were incubated with IL-1β (100 pg/ml) and the compounds of interest for 48 h, and cell culture medium was collected thereafter. Untreated cells were used as control, and PGE_2 levels determined by ELISA were quantified against IL-1β –treated cells. Values are mean + SEM, n = 3-4, ** = $p < 0.01$.

Fig 1

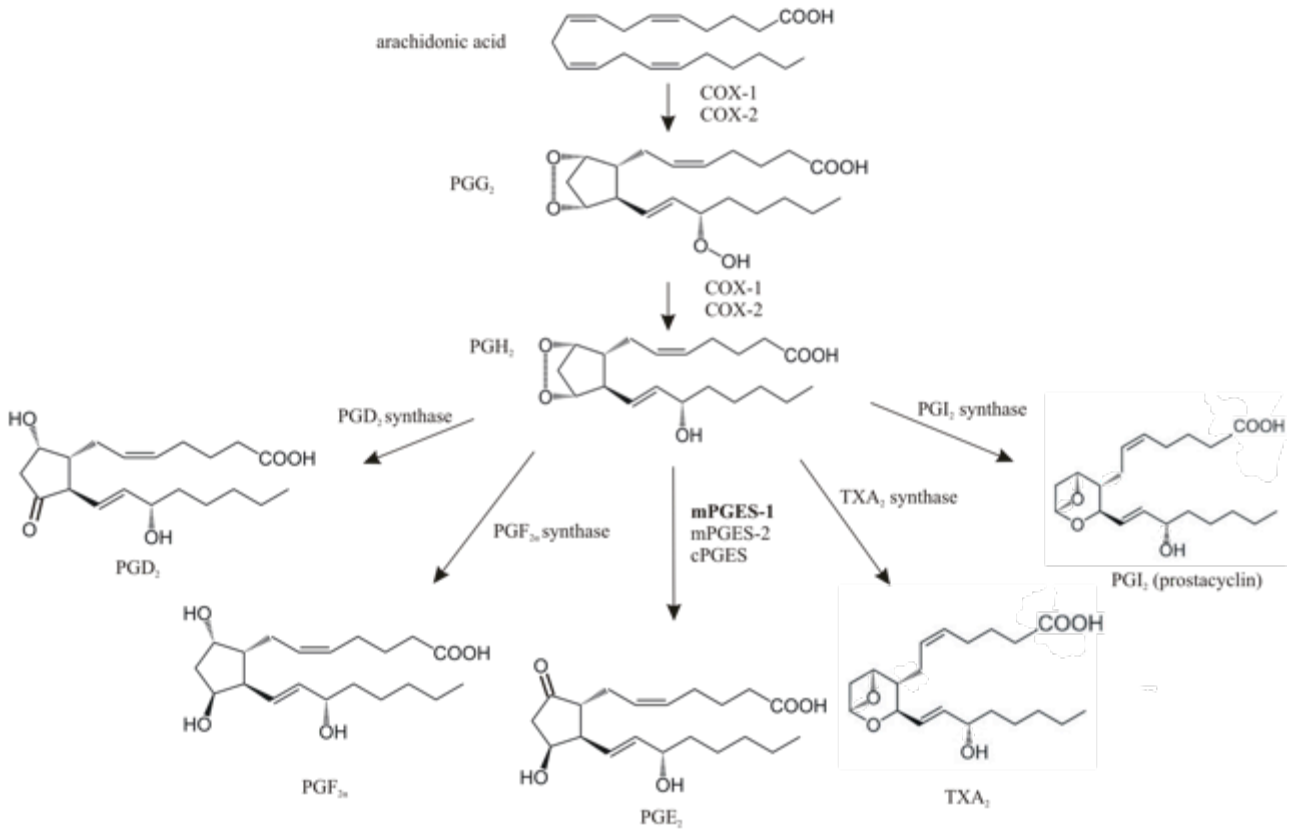

arachidonic acid
COOH
COX-1
COX-2
PGG2
COOH
OOH
COX-1
COX-2
PGH2
COOH
OH
PGD2 synthase
PGI2 synthase
TXA2 synthase
PGF2α synthase
mPGES-1
mPGES-2
cPGES
HO
COOH
O
OH
PGD2
COOH
O
O
OH
PGI2 (prostacyclin)
HO
COOH
HO
OH
PGF2α
O
COOH
HO
OH
PGE2
COOH
O
O
OH
TXA2

Fig 2

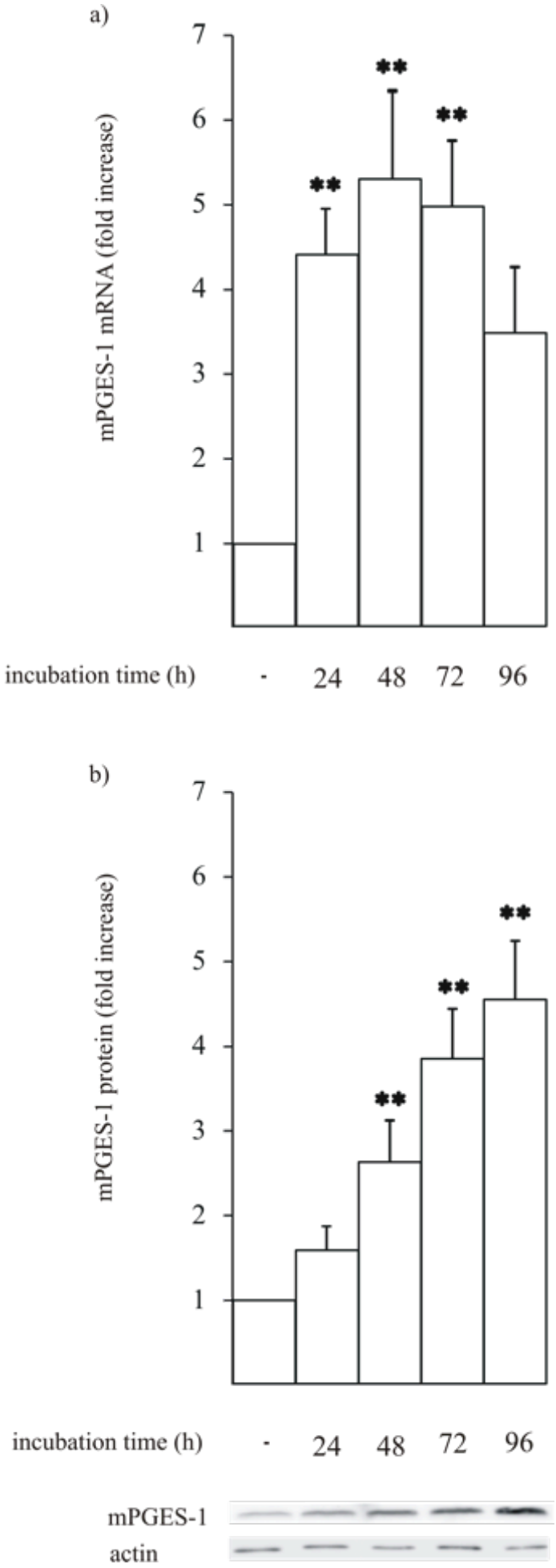
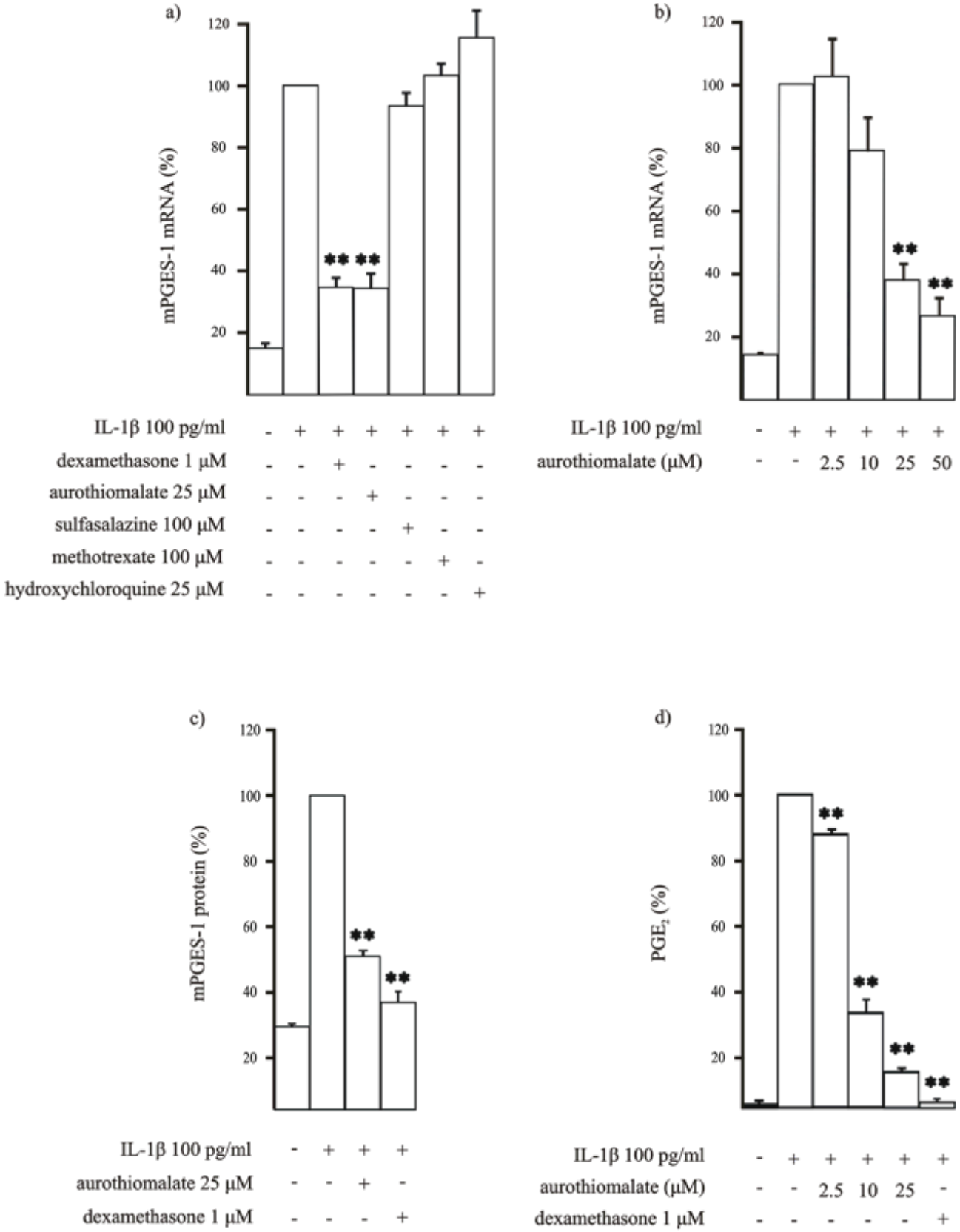

a)
mPGES-1 mRNA (fold increase)
7
6
5
4
3
2
1
**
**
**
incubation time (h)
-
24
48
72
96
b)
mPGES-1 protein (fold increase)
7
6
5
4
3
2
1
**
**
**
incubation time (h)
-
24
48
72
96
mPGES-1
actin

Fig 3


a)
mPGES-1 mRNA (%)
120
100
80
60
40
20
**
**
IL-1β 100 pg/ml - + + + + + +
dexamethasone 1 μM - - + - - - -
aurothiomalate 25 μM - - - + - - -
sulfasalazine 100 μM - - - - + - -
methotrexate 100 μM - - - - - + -
hydroxychloroquine 25 μM - - - - - - +
b)
mPGES-1 mRNA (%)
120
100
80
60
40
20
**
**
IL-1β 100 pg/ml - + + + + +
aurothiomalate (μM) - - 2.5 10 25 50
c)
mPGES-1 protein (%)
120
100
80
60
40
20
**
**
IL-1β 100 pg/ml - + + +
aurothiomalate 25 μM - - + -
dexamethasone 1 μM - - - +
mPGES-1
actin
d)
PGE2 (%)
120
100
80
60
40
20
**
**
**
**
IL-1β 100 pg/ml - + + + + +
aurothiomalate (μM) - - 2.5 10 25 -
dexamethasone 1 μM - - - - - +